



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q 1/68	A2	(11) International Publication Number: WO 00/05410 (43) International Publication Date: 3 February 2000 (03.02.00)
<p>(21) International Application Number: PCT/IL99/00404</p> <p>(22) International Filing Date: 22 July 1999 (22.07.99)</p> <p>(30) Priority Data: 125456 22 July 1998 (22.07.98) IL 128017 12 January 1999 (12.01.99) IL</p> <p>(71) Applicant (for all designated States except US): TECHNION RESEARCH AND DEVELOPMENT FOUNDATION LTD. [IL/IL]; Senate House, Technion City, Park Gootwirt, 32000 Haifa (IL).</p> <p>(72) Inventor; and (75) Inventor/Applicant (for US only): ARONHEIM, Ami [IL/IL]; Hagavia 7, 30500 Binyamina (IL).</p> <p>(74) Agent: REINHOLD COHN AND PARTNERS; P.O. Box 4060, 61040 Tel Aviv (IL).</p>	<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>	
(54) Title: METHOD FOR DETECTING PROTEIN-PROTEIN INTERACTIONS AND A KIT THEREFOR		
(57) Abstract		
<p>A method for detecting an interaction between two tester proteins. In a cell incapable of activating a Ras protein, two nucleic acid sequences are expressed. One sequence encodes for a fusion protein comprising a mutant Ras protein incapable of localizing at the cell membrane and not requiring an exchange factor fused to one of the tester proteins. The other sequence encodes for the other tester protein fused to a plasma membrane localization domain. An interaction between the two fusion proteins leads to the expression of a functional Ras protein that is detected as an altered cell phenotype.</p>	<p>A.</p> <p>B.</p>	

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

METHOD FOR DETECTING PROTEIN-PROTEIN INTERACTIONS AND A KIT THEREFOR

FIELD OF THE INVENTION

The present invention relates generally to molecular biology and more specifically to a method for identifying protein pairs involved in protein-protein interactions.

5 BACKGROUND OF THE INVENTION

The phrase "*protein-protein interactions*" refers to the ability of two protein molecules to bind to each other so as to form a complex. Such protein-protein interactions are involved in a large variety of biological processes including, for example, signal transduction pathways, enzyme- substrate
10 interactions, viral adhesions and the formation of antibody-antigen complexes.

Many proteins are capable of interacting with a number of other proteins. Identifying and characterizing such interactions are highly important in understanding biological mechanisms, signal transduction pathways, etc. in characterizing the molecular basis of various diseases as disorders and in the
15 design of therapies.

A defect in a protein preventing it from participating in a protein-protein interaction can, as may be appreciated, have deleterious effects on a cell.

The ability to identify and characterize protein-protein interactions permits the identification of the defects in such interactions associated with a diseased state. The identification of such defects provides a target for potential therapies to cure or ameliorate the disease. In addition, the identification and characterization of protein-protein interactions provides a means to screen for drugs that alter the interaction. Such drugs can be useful, for example, to treat a disease caused, at least in part, by an aberrant protein-protein interaction.

Methods for assaying protein-protein interactions have been reviewed in Allen *et al.*, *Trends Biochem. Sci.*, 20:511-516, 1995.

Proteins involved in a protein-protein interaction can be identified by detecting the presence of the protein complex in a cell or in a body fluid, and purifying the proteins forming the complex by biochemical methods. Such methods of isolation, however, are extremely tedious, particularly when the protein is expressed at low levels or if only a few cells express the protein. Immobilization of proteins on membrane filters has more recently lead to the development of filter based assays using proteins translated from cDNA molecules obtained, for example, from phage. However, the filter based assays, while being more sensitive, are also often very tedious.

A genetic method of identifying protein-protein interactions has also been developed (Fields *et al.*, *Nature*, 340:245-246, 1989). In this method, known as the "two hybrid assay", one protein is fused to a DNA binding domain (typically from the Gal4 protein) while another protein is fused to a strong transcription activation domain. Binding of the two proteins inside a cell thus generates a functional transcription factor that is detected by a change in phenotype of the cell due to the expression of genes whose transcription is under the control of Gal4 DNA elements. The two hybrid system however, suffers from several limitations.

First, protein pairs in which one of the proteins possesses transcriptional activity on its own, obviously cannot be analyzed. This includes *bona fide* transcription factors as well as proteins containing domains that fortuitously interact with the transcription machinery. Another limitation of the two hybrid system results from
5 the toxicity of many proteins, for example certain homeodomain proteins and cell cycle regulators, when expressed in the nucleus. Furthermore, the two hybrid system produces false positive or false negative results when one of the proteins undergoes a conformational change in the nucleus.

Another genetic method, the "*Sos Recruitment System*" (SRS) has also
10 been described (Aronheim, A., *Mol. Cell. Biol.*, 17:3094-3102, 1997). This method is based on the observation that localization of the protein hSos (the Ras guanyl nucleotide exchange factor) at the plasma membrane is essential for activating the Ras pathway and is therefore essential for viability. A yeast strain, such as *cdc25-2*, containing a temperature sensitive allele of Cdc25, (a yeast
15 homologue of hSos) is thus viable only at the permissive temperature (24°C). In the SRS system, a first protein (the bait protein) is fused to hSos while a second protein (the prey protein) is fused to a membrane localization domain. A protein-protein interaction between the bait and prey proteins localizes hSos at the plasma membrane. This complements the Cdc25 mutation which is detected as
20 cell growth at the restrictive temperature (36°C). However, the SRS also exhibits several limitations. First, about 20-30% of all bait proteins fused to hSos result in prey-independent complementation of Cdc25, a fact which yields a relatively high unspecific background signal ("*noise*"). Another limitation of the SRS system is that the effector part of the hSos is relatively large (150 Kda). This
25 tends to complicate the fusion to hSos of both large bait proteins as well as short bait proteins.

Another problem of the SRS system is due to the fact that Ras encoded proteins are able to bypass the Cdc25 mutation because the yeast GTPase

activating proteins (IRA genes) hydrolyze GTP bound to mammalian Ras proteins rather inefficiently thus leaving the Ras proteins in their active GTP-bound form.

SUMMARY OF THE INVENTION

5 The present invention makes use of the fact that in order for it to function, Ras needs to be localized at the plasma membrane. This localization normally occurs via the covalent attachment of a lipid moiety to cysteine 186 that anchors Ras at the membrane. Ras contains a consensus CAAX box located at the C-terminal end which undergoes farnesylation and subsequently palmitoylation.

10 A Ras lacking the farnesylation box (CAAX) is non-functional since it cannot be localized at the membrane. The present invention thus makes use of cells with a Ras that is mutated such that it cannot be localized at the membrane, e.g. lacking the farnesylation box or having a mutation therein. These cells are "engineered" such that they express two fusion proteins, one fusion protein comprising a first

15 protein (referred to herein at times as the "bait") and a Ras protein which is mutated such that it cannot bind to the plasma membrane, and another fusion protein which comprises a second protein (referred to herein at times as the "prey") and a membrane localization domain. If the bait binds the prey then the Ras fused to the prey becomes localized at the membrane and can thereby

20 function.

Fig. 1 shows a schematic representation of the invention. In panel A, a cell incapable of expressing a functional Ras is made to express a Ras that cannot be localized at the membrane (and is thus non-functional) fused to a bait protein. A putative prey protein has been localized at the plasma membrane. A

25 protein-protein interaction between the prey and bait proteins (panel B) localizes Ras at the plasma membrane. This produces a functional Ras that is detected as a phenotypic change in the cell.

This Ras Recruiting System (RRS) has several advantages over the SRS system:

1. The Ras protein is relatively small, thereby overcoming several of the technical limitations and practical problems posed by the large size of Sos as described above.
2. The RRS system exhibits substantially less false positive results, as compared to the SRS, with mammalian cDNA expression library screens and therefore represents a more efficient system for characterizing interacting proteins.

The invention thus provides a method for identifying a protein-protein interaction between a first protein and a second protein comprising the steps of:

- (a) expressing in a cell which is incapable of activating a Ras protein;
 - (aa) a first nucleic acid sequence encoding a first fusion protein, said first fusion protein comprising a Ras protein mutated such that it cannot localize at the cell membrane and does not require an exchange factor fused to said first protein; and
 - (ab) a second nucleic acid sequence encoding a second fusion protein said second fusion protein, comprising said second protein fused to a plasma membrane localization domain; and
- (b) determining whether there is a phenotypic expression of a functional Ras protein in said cell, the presence of a functional Ras protein in said cell indicating a protein-protein interaction between said first protein and said second protein.

In a preferred embodiment of the invention, the mutated Ras protein, which forms part of the fusion proteins encoded by the first nucleic acid sequence, lacks a farnesylation box.

In another preferred embodiment of the invention, the cell incapable of expressing a functional Ras is of the yeast strain cdc 25-2. The Ras of this cell is

non-functional at the restrictive temperature (36°C) due to a lack of a functional guanyl nucleotide exchange factor. Production of a functional Ras in these cells by an interaction between a bait protein and a prey protein according to the invention is detected as growth independent of a functional exchange factor at
5 such a restrictive temperature, e.g. at about 33-37°C, typically at about 36°C.

The method of the invention is useful for screening of gene libraries to fixed expression products that interact with a specific protein. As already pointed out above, determining protein-protein interaction may be highly important for drug development. In addition, determining such interaction may serve as a
10 diagnostic trend; for example, a specific pattern of interaction of one protein with others, may serve as an indication of a normal or a mutated protein.

The method of the invention lends itself also to application in high throughput screening techniques. Cells may be automatically supplemented with DNA constructs, e.g. plasmids, under conditions in which such constructs will be
15 internalized by the cells and then screened automatically for such with a Ras phenotype expression.

The invention also provides a system for use in determining whether a first protein is capable of binding to a second protein, comprising:

- (a) a culture of cells incapable of activating a Ras protein;
- 20 (b) a first nucleic acid vector, for inserting therein a DNA sequence encoding a first fusion protein which comprises a Ras protein mutated such that it cannot localize to the cell membrane and does not require an exchange factor and said first protein;
- (c) a second nucleic acid vector, which may be the same or different
25 than said first nucleic acid vector, for inserting therein a DNA sequence encoding a second fusion protein which comprises said second protein and a plasma membrane localization domain;

(d) reagents and devices for transfecting the cells with said first and said second nucleic acid;

(e) a monitoring arrangement for monitoring phenotypic Ras expression in said cells.

5 Also provided by the invention is a kit comprising some or all of the constituents of the above system.

Localization of a mammalian Ras fused to a bait of interest at the plasma membrane through a protein-protein interaction in a temperature sensitive mutant such as cdc25-2 permits growth at 36°C. Ras localization can also complement a
10 temperature sensitive mutant yeast strain that is defective in its exchange factor. hSos, however, complements the yeast cdc25 mutant strain only when expressed in the membrane, but Ras^{ts} fails to do so in the yeast Ras^{ts} mutant strain, which is defective in its Ras. This eliminates isolation of mammalian Ras exchange factors in a library screen.

15 As provided by the invention, mammalian expression vectors comprising regions encoding for interacting protein partners used in the yeast RRS system can be co-transfected with a reporter gene such as a chloramphenicol acetyl transferase (CAT) or luciferase gene under the control of either AP-1 responsive elements or Ras responsive elements. Cultured mammalian cells expressing these
20 plasmids allow a protein-protein interaction known to occur in yeast to be quantitatively detected in mammalian cells by monitoring the enzymatic activity of the reporter gene following the protein-protein interaction and recruitment of activated Ras to the plasma membrane. Use of reporter genes in mammalian cells allows direct evidence of a protein-protein interaction initially identified in yeast
25 and improved assessment of drug effectiveness directly in mammalian cells.

The invention also provides a positive control for proteins for which a protein partner is not detected in a library screening as described above. In cells not expressing a functional Ras, a fusion protein is expressed comprising a

functional Ras fused to two bait proteins. The first bait ("*the tester bait*") is the protein having no known protein partners while the second bait ("*the control bait*") is a protein having a known protein partner. The ability of the control bait moiety to bind its known prey is determined. A protein-protein reaction occurring
5 between the control bait moiety and its prey demonstrates that the gene for the fusion moiety is expressed at adequate levels that the Ras moiety of the fusion protein is functional, and that the Ras pathway is intact. This would provide genetic evidence that screening with the tester bait is potentially possible and worthwhile.

10 The fusion of a tester bait and a control bait to a single Ras molecule may be used for mapping the amino acids involved in an interaction between the tester bait and its prey. The tester bait is subjected to random mutagenesis and inserted fused in frame with the control bait. The tester bait DNA is inserted in frame with that of Ras and the control bait and expressed, for example, in *cdc25-2* cells of
15 one mating type. The cells are then mated with cells of the opposite mating type expressing either the prey of the tester bait or the control bait. Cells able to grow with the control bait but not with the tester bait prey indicate the integration of a mutation that affects the tester bait prey binding that is not due to a frame shift or nonsense mutation.

20 Similarly this embodiment may also be used for screening drugs for the ability to inhibit an interaction between a tester bait and its prey while not inhibiting an interaction between the control bait and its prey.

BRIEF DESCRIPTION OF THE DRAWINGS:

25 In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting examples only, with reference to the accompanying drawings, in which:

Fig. 1 shows a schematic diagram of the RRS system;

Fig. 2 shows the complementation of the *cdc25-2* mutation through protein-protein interactions. A. – p110-p85 interaction. B. – Jun-Fos interaction. C. – Grb2-hSos-C.

5 Fig. 3 shows the complementation of the *cdc25-2* mutation by 5'Sos-Pak bait in a prey-independent manner.

Fig. 4 shows the interaction of Pak65 regulatory domain (Pak) with known protein partners using the RRS system. A. – Pak65 interaction with different SH3 containing proteins; B. – Pak65 interaction with Rac1 mutants.

10 Fig. 5 shows the isolation of novel protein interaction using Pak65 regulatory domain as a bait and the RRS system.

EXAMPLES

MATERIALS AND METHODS

15 Plasmids and constructs

Ras plasmid construction: Ras-specific oligonucleotides were designed to generate a Ras fragment corresponding to amino acids 2-185. Ras DNA fragment was designed to contain 5'-AAG CTT CCC GGG ACC ATG to provide HindIII SmaI NcoI restriction sites to allow N-terminal fusion and either 5'-TGG ATC
20 CTC or 5'-GGA ATT CTC to provide either a BamHI or EcoRI restriction sites respectively to allow C-terminal fusion.

ADNS expression vectors: p110-Ras Δ F-Wt., p110-Ras(61) Δ F encode for p110 β amino acids 31 to 150 fused to either wild type Ras or activated Ras(L61) respectively devoid of its CAAX box. JZ-Ras Δ F-Wt., JZ-Ras(61) Δ F encode for
25 c-Jun DNA binding domain amino acids 249-331 fused to Ras as described above. Sos-C-Ras(61) Δ F encodes for hSos C-terminal region amino acids 1066-1333 fused to activated Ras(L61) devoid of its CAAX box. PAK65, amino

acids 3-215, was fused to Ras(61) Δ F. The DNA fragments of p110, JZ, Sos-C and PAK65 were fused to Ras(L61) Δ F into HindIII-SmaI. JDP2 cDNA was fused to Ras to its C-terminal domain using EcoRI-XhoI restriction and ligated by three fragments ligation into ADNS HindIII-SalI to generate ADNS-Ras(61) Δ F-JDP2.

5 Yes2 expression vectors: Yes-M, Yes-M-p85, Yes-M-Fos are as described, Yes-M-Grb2 and Yes-M*-Grb2 encode for full length Grb2 fused to either myristoylation (M) or myristoylated defective sequence. Yes-M-PLC γ 2 corresponds to amino acids 405-1252 of PLC γ 2 fused to Yes-M. Yes-M-p85(SH3) corresponds to amino acids 2-84 of p85. Yes-M-GAP(SH3)
10 corresponds to amino acids 262-345 of mammalian GAP.

The different Rac1 plasmids were constructed into pYes2 expression plasmid (Invitrogen Inc.).

Yeast growth and manipulations

15 Conventional yeast transfection and manipulation protocols were used. Cells were plated on either glucose minimal medium containing the relevant amino acids, 2% glucose, 0.5% NH₄SO₄, 0.17% yeast extract and 4% agar or galactose glucose. YPD medium contains: 1% yeast extract, 2% bacto peptone, 2% glucose. Replica plating was performed with homemade disposable velvet
20 replica plating.

Library screening

Screening of the library was performed stepwise. First the bait was cotransfected into cdc25-2 cells together with Yes(trp)-mGAP expression
25 plasmid. Transformants were selected on glucose minimal medium lacking the amino acids leucine and tryptophan at 24°C. Subsequently, 3 ml culture was grown in liquid at 24°C overnight and used to inoculate 200 ml medium for an

additional 12 hours. Cells were pelleted and resuspended in 200 ml YPD medium for 3-5 hours at 24°C and used directly for transfection with 20-40 µg of library plasmid DNA. Cells were plated on about twenty 10 cm plates resulting in 5,000-10,000 transformants/plate.

5

Example 1 Use of RRS to detect an interaction between cytoplasmic protein pairs

The interaction between two cytoplasmic proteins, the phosphatidylinositol-3-phosphate kinase subunits p110 and p85 was tested using the RRS system (Fig. 2A). The p85 interacting domain of p110 was fused to either activated or wild type mammalian cytoplasmic Ras p110-Ras(61)ΔF and p110RasΔF-Wt., respectively. Plasmids were cotransfected in different combinations with either the Yes2 empty expression vector or a plasmid encoding for myristoylated p85 (Yes-M or Yes-M-p85 respectively) into Cdc25-2 yeast cells and grown at 24°C on glucose minimal medium supplemented with the appropriate amino acids and bases. Four independent transformants were grown at 24°C (left panel), replica plated onto appropriately supplemented galactose minimal plates and grown at 36°C (right panel). Constructs used are described in Material and Methods. Only transformants expressing both the p110-Ras fusion protein and membrane anchored p85 were able to grow at 36°C. No significant difference was observed when p110 was fused to either the wild type or activated cytoplasmic Ras.

25

Example 2 Use of RRS to detect an interaction between nuclear protein pairs

The interaction of two nuclear proteins c-Jun and c-Fos was tested using the RRS (Fig. 2B). The DNA binding domain of c-Jun was fused to either

30

activated or wild type mammalian cytoplasmic Ras (JZ-Ras(61) Δ F and JZ-Ras Δ F-Wt. respectively). These plasmids were cotransfected with their Yes2 empty expression vector or a plasmid encoding myristoylated c-Fos (Yes-M or Yes-M-Fos) to cdc25-2 yeast cells and grown at 24°C (Fig. 2B, left panel).

- 5 Transformants were isolated and tested for their ability to grow at the restrictive temperature 36°C (Fig. 2B, right panel). Only transformants expressing both the c-Jun-Ras fusion protein and membrane anchored c-Fos were able to grow at the restrictive temperature.

10 **Example 3 Use of RRS to detect an interaction between hSos C-terminal domain and Crb2**

- The interaction of the C-terminal region of hSos (containing the proline rich region) with the adaptor protein Grb2 was tested (Fig. 2C). hSos C-terminal domain was fused to cytoplasmic activated mammalian Ras (Soc-C-Ras(61) Δ F). This plasmid was cotransfected with either empty vector (Yes-M), membrane anchored Grb2 (Yes-M-Grb2) or cytoplasmic Grb2 (Yes-M*-Grb2) to cdc25-2 yeast cells and grown at 24°C on appropriate medium (Fig. 2C, left panel). Transformants were isolated and tested for their ability to grow at 36°C. Only transformants expressing both the Soc C-terminal fusion protein and membrane anchored Grb2 were able to grow at 36°C (Fig. 2C, right panel). RRS is thus superior to the SRS system for identification of Soc C-terminal interacting proteins, since this domain confers an inhibitory activity on Sos function.

25

Example 4 Pak65

As described above, one of the problems associated with the SRS system is prey independent-activity of hSos fused to some proteins. One such

protein is the p21 Activated Kinase, Pak65. Pak65 activates Rac and Cdc42 by binding them in a GTP dependent manner. Despite considerable efforts, little information is available concerning the role of Pak65 in signal transduction. The Pak65 regulatory domain contains two protein modules known to mediate

5 protein-protein interactions: a proline rich region that binds SH3 containing proteins and a Cdc42/Rac interacting binding domain (CRIB) found in a number of proteins that bind the small GTPase proteins from the Rho family. In order to gain insight into Pak65 function, proteins binding to the Pak65 regulatory domain using the SRS system were screened (Fig. 3). The Pak65

10 regulatory domain was fused to 5'Sos and expressed in *cdc25-2* yeast cells. Transformants expressing 5'Sos-Pak exhibit efficient growth even in the absence of a protein partner (Fig. 3). This renders the SRS system ineffective for screening with the Pak65 bait. However, *cdc25-2* yeast transformants expressing the Pak65 regulatory domain fused to the cytoplasmic activated Ras

15 (Pak-Ras(61) Δ F) were unable to grow at the 36°C (Fig. 4A, right panel) and therefore the Pak65 bait could be used for further analysis.

Example 5 Use of RRS for isolation of novel protein interactions

The Pak65 regulatory domain was used as a bait to screen a rat pituitary

20 cDNA expression library fused to a membrane localization sequence. Expression of the library cDNA insert plasmid is under the control of the GAL1 promoter. About 500,000 independent transformants were screened for interaction with Pak-Ras(61) Δ F bait in the presence of a plasmid encoding for mammalian GAP. Five clones were isolated that exhibited efficient cell growth at 36°C only when

25 grown under galactose inducing conditions. Plasmids derived from these yeast clones were cotransfected with either the original Pak65 bait (Pak-Ras(61) Δ F) or an irrelevant bait (Ras(61) Δ F-JDP2). DNA plasmids from four independent

clones encoded proteins that exhibited a specific interaction with Pak65 bait resulting in efficient growth at 36°C but did not grow when expressed with the irrelevant bait at 36°C (Fig. 5). Only one plasmid DNA produced efficient growth at 36°C with both the specific and non-specific baits. Sequence analysis of this
5 clone identified it as a Sos-homologue which would be expected to activate yeast Ras and bypass the requirement for mammalian Ras translocation.

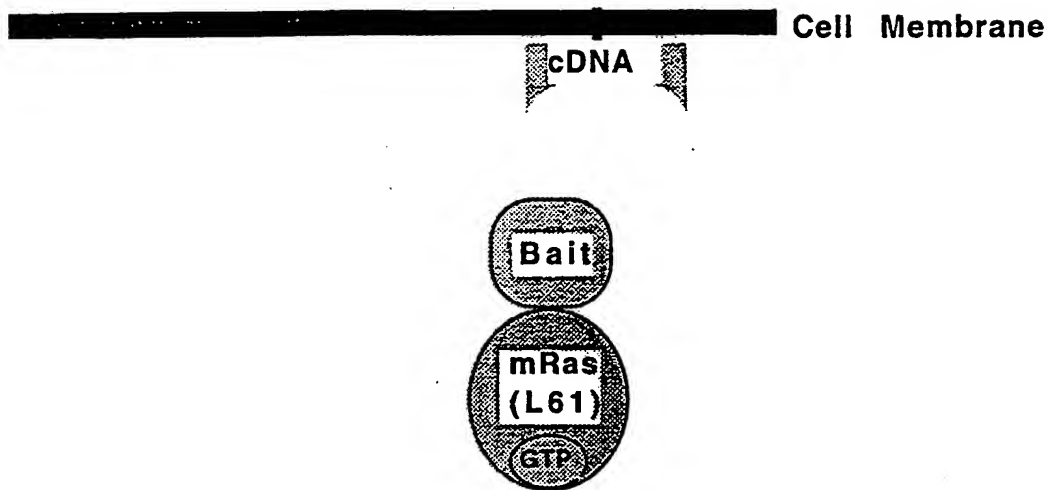
CLAIMS:

1. A method for identifying a protein-protein interaction between a first protein and a second protein comprising the steps of:
 - (a) expressing in a cell which is incapable of activating a Ras protein;
 - 5 (aa) a first nucleic acid sequence encoding a first fusion protein, said first fusion protein comprising a Ras protein mutated such that it cannot localize at the cell membrane and does not require an exchange factor fused to said first protein; and
 - (ab) a second nucleic acid sequence encoding a second fusion protein
10 said second fusion protein, comprising said second protein fused to a plasma membrane localization domain; and
 - (b) determining whether there is a phenotypic expression of a functional Ras protein in said cell, the presence of a functional Ras protein in said cell indicating a protein-protein interaction between said first protein
15 and said second protein.
2. The method of Claim 1, wherein the mutated Ras protein comprised in the fusion protein encoded by said first nucleic acid sequence, lacks a farnesylation box.
3. The method of Claim 1 or 2, wherein said cell is a yeast cell.
- 20 4. The method of Claim 3, wherein said yeast cell is a *Saccharomyces cerevisiae* cdc25-2 cell.
5. The method of Claim 4, wherein the presence of a functional Ras protein in said cell is detected by cell growth at 33-37°C.
6. The method of Claim 1, wherein said plasma membrane localization
25 domain is a myristoylation signal.
7. A system for use in determining whether a first protein is capable of binding to a second protein, comprising:

- (a) a culture of cells incapable of activating a Ras protein;
 - (b) a first nucleic acid vector, for inserting therein a DNA sequence encoding a first fusion protein which comprises a Ras protein mutated such that it cannot localize to the cell membrane and does not require an exchange factor and
5 said first protein;
 - (c) a second nucleic acid vector, which may be the same or different than said first nucleic acid vector, for inserting therein a DNA sequence encoding a second fusion protein which comprises said second protein and a plasma membrane localization domain;
 - 10 (d) reagents and devices for transfecting the cells with said first and said second nucleic acid;
 - (e) a monitoring arrangement for monitoring phenotypic Ras expression in said cells.
8. A kit comprising components of the system of Claim 7.

1 / 5

A.



B.

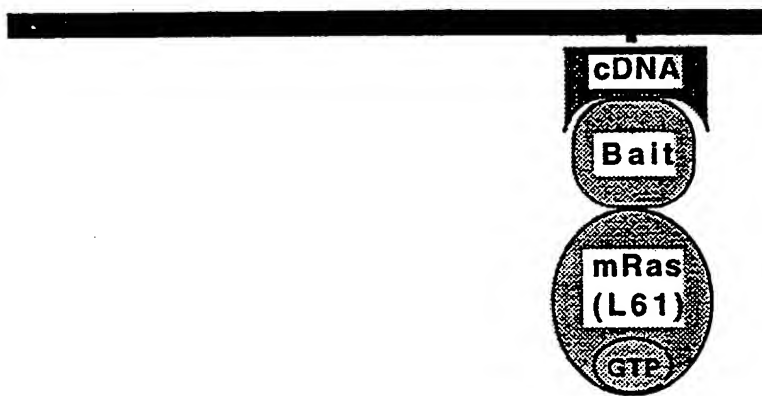


Fig. 1

2/5

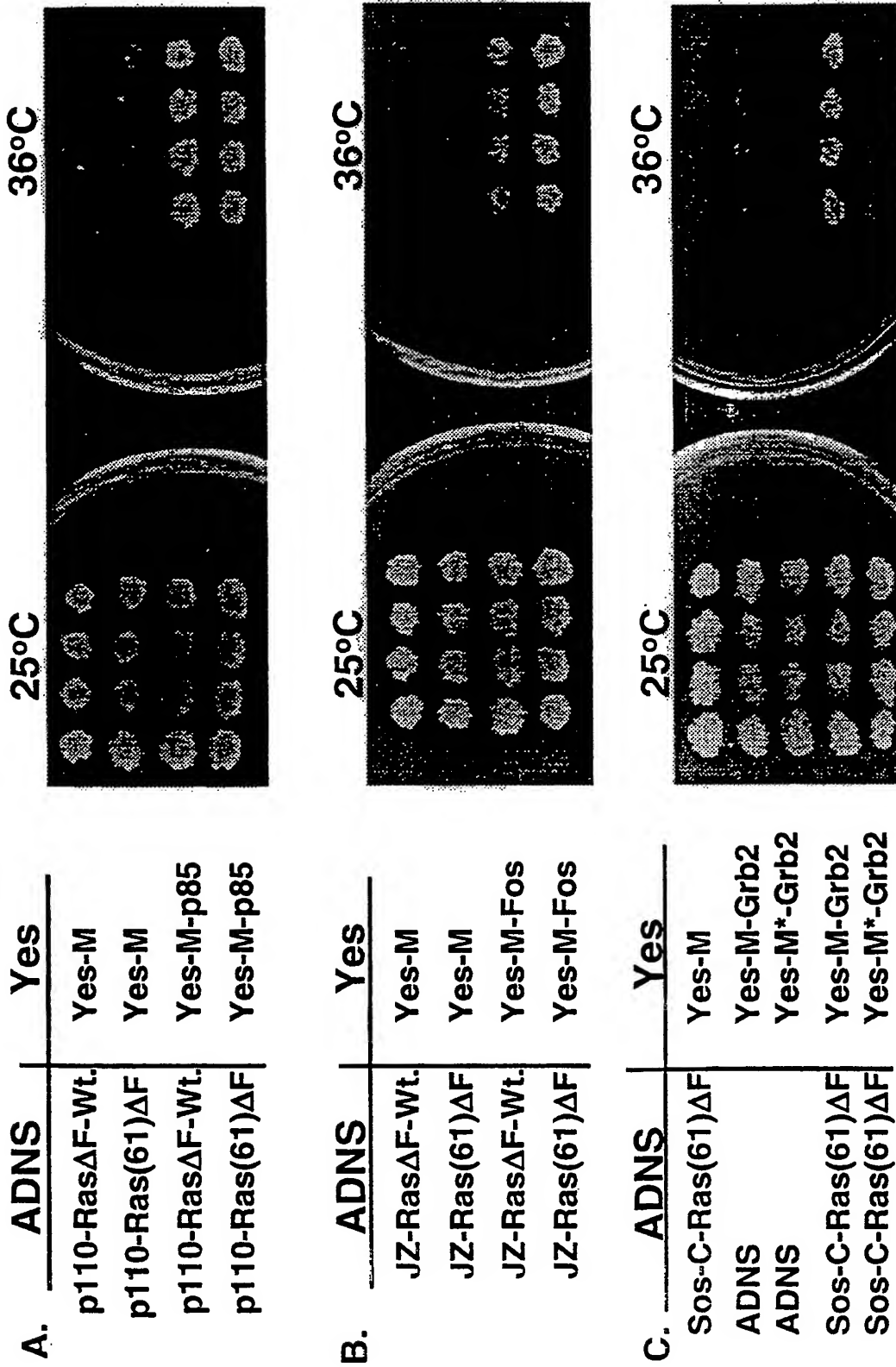


Fig. 2

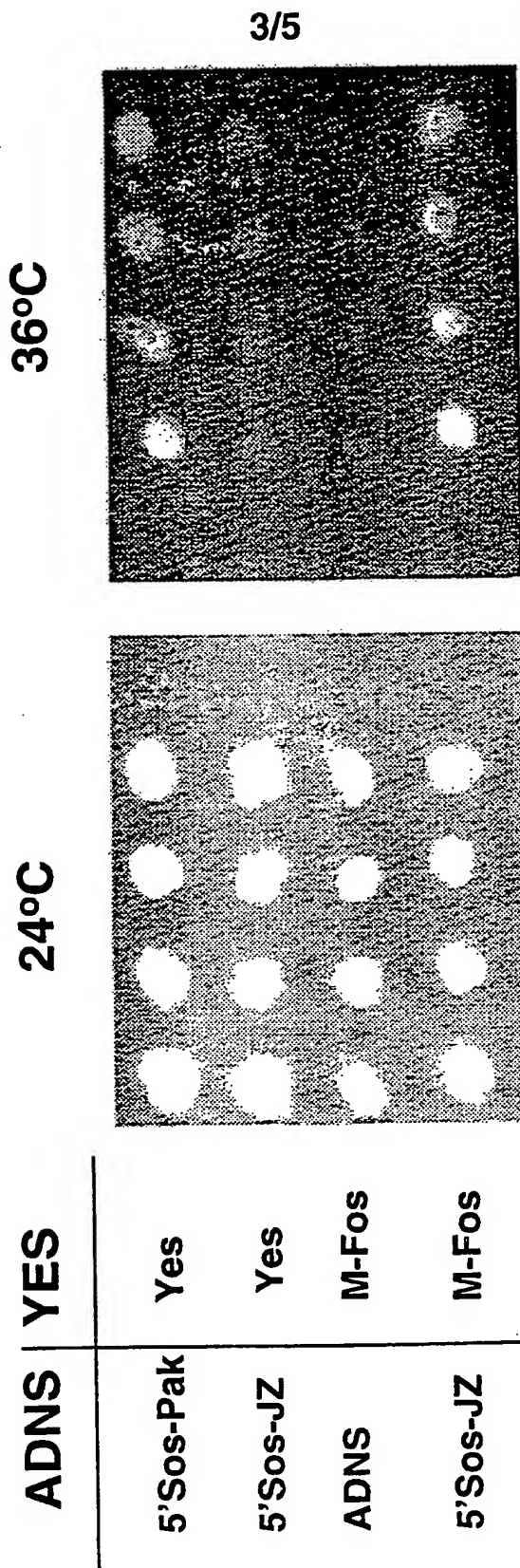


Fig. 3

4/5

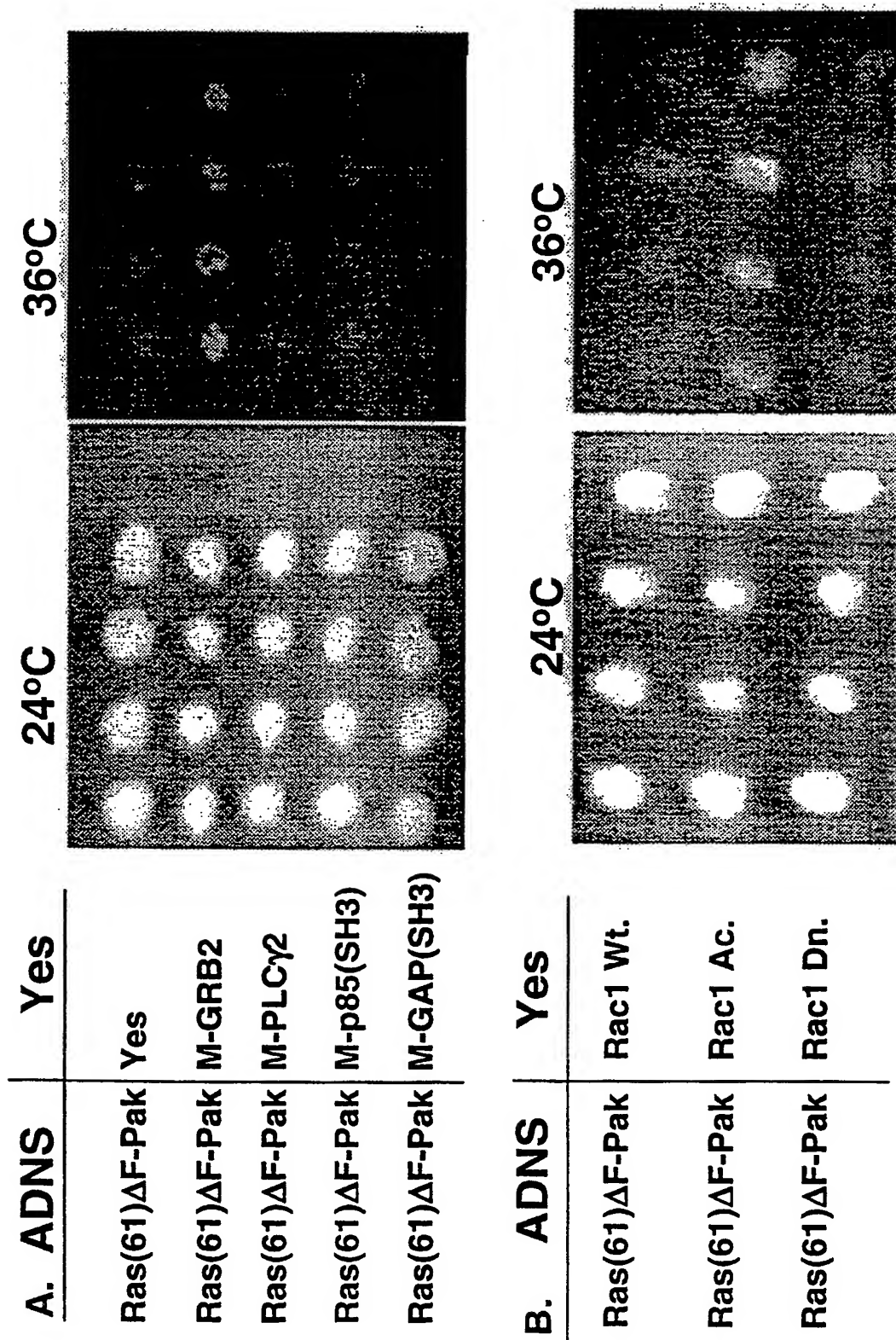


Fig. 4

5/5

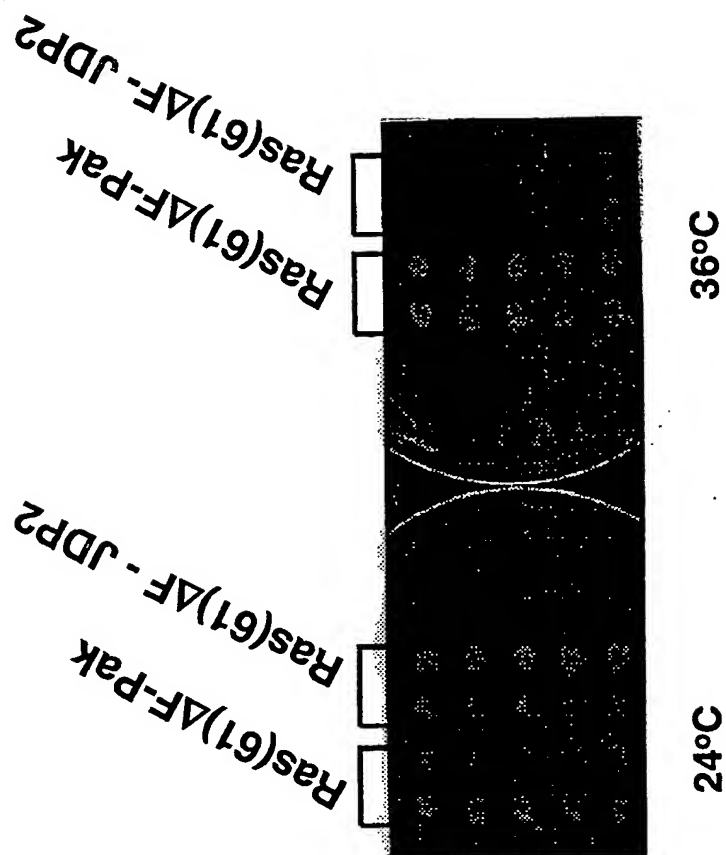


Fig 5

Yes-M	SEQUENCE SIMILARITY
#1	CHP
#2	CHP
#5	CHP
#6	unknown
#8	Sos

THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)